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**Nutrient and secondary metabolite concentrations in a savanna are
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Peter F. Scogings

Department of Agriculture, University of Zululand, Private Bag X1001, KwaDlangezwa
3886, South Africa

e-mail: scogingsp@unizulu.ac.za; Phone: +27.35.902.6063; Fax: +27.35.902.6056

Joakim Hjältén

Department of Wildlife, Fish, and Environmental Studies, Swedish University of
Agricultural Sciences, Umeå, Sweden

Christina Skarpe

Department of Forestry and Wildlife Management, Hedmark University College,
Koppang, Norway

Dawood Hattas

Department of Biological Sciences, University of Cape Town, Rondebosch, South
Africa

26

27 Alpheus Zobolo

28 Department of Botany, University of Zululand, KwaDlangezwa, South Africa

29

30 Luthando Dziba

31 Natural Resources and Environment, Council for Scientific & Industrial Research,

32 Pretoria, South Africa

33

34 Tuulikki Rooke

35 Swedish Environmental Protection Agency, Stockholm, Sweden

36

37

38 **Abstract** Carbon-based secondary metabolites (CBSMs) such as tannins are assumed
39 to function as plant defences against herbivores. CBSMs are thought to be inversely
40 related to growth rate and nutrient concentrations because a physiological trade-off
41 exists between cellular growth and differentiation, but CBSM concentrations can be
42 altered by herbivory-induced changes in the trade-off. We predicted that a significant
43 interaction exists between herbivory and growth phase, such that the effects of large
44 herbivores (or their exclusion) on nutrient or CBSM concentrations are greatest during
45 phases of rapid shoot or leaf growth. Leaf samples were collected during phases of
46 different growth rate from six woody species four years after establishment of a large-
47 scale long-term herbivore exclusion experiment in Kruger National Park, South Africa.
48 Samples were analysed for N, P, condensed tannins and total phenolics. Interactions
49 between growth phase and herbivores were rare. However, the assumption that elevated
50 nutrients and reduced CBSMs occur during fast phases of growth was supported by four

species (consistent with the growth-differentiation balance hypothesis), but not the other two. Large herbivores generally did not affect nutrients, but CBSMs in four species were reduced by large herbivores other than elephants, while CBSMs in two species were reduced by elephants. Carbon limitation ultimately prevailed among woody plants taller than 2 m under long-term browsing. Large herbivores and plant growth phase are independent and important determinants of nutrients or CBSMs in African savannas, but the effects depend on the interacting assemblages of species, which poses challenges to the application of current general hypotheses of plant defence.

Key words Elephant, Exclusion experiment, Herbivore, Phenology, Plant defence

Introduction

Plant-herbivore interactions have profound impacts on ecosystems (Pastor et al. 2006). On the plant scale, several traits affect herbivore food choice, such as plant secondary metabolite and nutrient concentrations, which affect digestibility (Skarpe and Hester 2008). Carbon-based secondary metabolites (CBSMs), such as tannins and phenols, are ecologically important because they are assumed to function as chemical defences that contribute to the herbivore-avoidance strategies of woody plants (Skarpe and Hester 2008; Barbehenn and Constabel 2011; Boeckler et al. 2011). Nutrients, such as N and P, contribute to a plant's overall value for herbivores (Mårell et al. 2005). However, these plant traits do not only vary among plant species, but also vary over time and in response to herbivory (Lindroth et al. 2007). Models that explain variations in CBSMs

predict that growth rate and nutrient concentration are inversely related to CBSM concentrations because a physiological trade-off exists between cellular growth and differentiation processes (Herms and Mattson 1992; le Bot et al. 2009). Furthermore, plants respond to herbivores by adjusting growth rate and, consequently, CBSM and nutrient concentrations (Skarpe and Hester 2008). While defoliation may induce defences in woody plants (Ward and Young 2002; Wessels et al. 2007), severe browsing (especially when twig biting is involved) has been reported to have the opposite effect by causing either increased N concentrations or reduced tannin concentrations in individual plants (du Toit et al. 1990; Danell et al. 2003; Persson et al. 2005). A browsing-induced switch to a vegetative state characterised by fast-growing, N-rich/C-poor tissues potentially increases a plant's risk of being browsed again (Fornara and du Toit 2007; Skarpe and Hester 2008). One explanation given for the positive feedback is preferential allocation of C to growth of new shoots rather than CBSMs (Fornara and du Toit 2007; Hrabar et al. 2009), which can be achieved when the root:shoot ratio is altered such that shoot growth increases to restore the ratio (Herms and Mattson 1992; Renton et al. 2007).

The responses of individual plants to years or decades of accumulated losses to browsing are not clearly predicted by current models of herbivore resistance. Browse-browser interactions are particularly relevant in Africa because of the abundance of large herbivores compared to other continents (Bond et al. 2001; Fritz and Loison 2006). Therefore, a critical component of research in African savannas is the effects that large herbivores have on woody plants, with the ultimate objective being the development of improved management policies (O'Keefe and Alard 2002). While numerous studies have measured browsing induced changes in either growth or CBSMs in savannas, few have considered the responses of both growth and CBSMs

simultaneously (Scogings et al. 2013b). Most of the latter studies have been short-term experiments using manual methods of simulated browsing applied to deciduous species. The majority of these studies have shown increased shoot growth and unchanged CBSM concentrations in response to browsing (Gowda 1997; Gadd et al. 2001; Katjiua and Ward 2006; Hrabar et al. 2009), but some have found increased shoot growth and either increased or decreased CBSM concentrations (Scogings and Macanda 2005; Rooke and Bergström 2007). The studies that considered both growth and nutrients or CBSMs under long-term browsing are equally inconclusive. They showed either decreased shoot or leaf growth with no change in CBSM concentrations (Zinn et al. 2007), or increased shoot growth with either unchanged CBSM concentrations, or increased nutrient concentrations coupled with decreased CBSM concentrations (Fornara and du Toit 2007, Hrabar et al. 2009). There is a need for further research into the effects of browsing on nutrient or CBSM concentrations among woody species in African savannas, which would contribute to determining the utility of models based on growth-differentiation trade-offs for understanding plant-herbivore dynamics in savannas.

The main aim of this paper was to determine how concentrations of foliar nutrients or CBSMs of woody species are affected by large herbivores and different phases of shoot / leaf growth in a semi-arid savanna. Savannas are characterised by alternating wet and dry seasons that are each several months long (Kutsch et al. 2008). Most subtropical savannas in Africa are semi-arid and water is the main limiting resource for plant growth for most of the year (Scholes et al. 2003; Jacobs et al. 2007). Nitrogen is most available early in the wet season when elevated soil water drives mineralization (Scholes et al. 2003). Shoot growth therefore occurs in brief phases during the wet season (Williams et al. 2009). Our primary underlying assumption was that changes in shoot / leaf growth rate during the wet season lead directly to differences

in foliar concentrations of nutrients or CBSMs (rather than foliar chemistry being directly affected by nutrient or water availability). We specifically assumed that during phases of fast shoot or leaf growth nutrient concentrations are highest, or CBSM concentrations are lowest, because of a trade-off between cellular growth and differentiation (Herms and Mattson 1992; Martz et al. 2010). We also explicitly assumed that browsing is capable of inducing defences (e.g., increase tannin concentrations) when CBSM concentrations are low and growth rate is high (Bryant et al. 1991; Scogings et al. 2011). We therefore hypothesised a significant interaction between browsing and growth phase, such that the effects of browsers on nutrient or CBSM concentrations are greatest during phases of rapid shoot or leaf growth. We tested our hypothesis by sampling six woody species during different growth phases in one wet season in a large-scale, long-term exclusion experiment in Kruger National Park, South Africa. The species were among the most abundant in the study area and are common elsewhere (Online Resource 1). Inclusion of several, commonly occurring species in the study enriched the study by providing an opportunity for species-specific responses to be discovered (Levick and Rogers 2008). In addition, the design of the exclusion experiment encouraged exploration of the roles of different assemblages of large herbivores (Jonsson et al. 2010). The landscape scale of our approach is useful for the management of extensive areas used for either biodiversity conservation or livestock production (Levick and Rogers 2008).

Materials and methods

Sampling

Experimental exclusion treatments (24° 58' S, 31° 46' E) were established adjacent to the Sabie River, Kruger National Park, South Africa in 2002 on the premise that exclusion of large herbivores (especially elephants) would allow their effects to be determined (O'Keefe and Alard 2002). Treatments could not be replicated because of the large scale needed for informing management decisions in extensive conservation areas comprising heterogeneous landscapes and mega-herbivores (Levick and Rogers 2008; Jonsson et al. 2010). One exclusion treatment excludes all herbivores the size of hares and larger (total exclusion; 70 ha), while a second exclusion treatment excludes elephants, but not other herbivores (partial exclusion; 44 ha). Although exclusion of giraffe from the partial exclusion treatment is unavoidable, they are not as abundant as elephants in the study area and their effect is assumed to be negligible. The two exclusion treatments are separated by a control area open to all large herbivores (no exclusion; 25 ha). Therefore, if allowing elephants has any additional effects on vegetation than allowing only other large herbivores, then it can be inferred that elephants are the main source of the differences between exclusion and control treatments (Jonsson et al. 2010). If excluding elephants shows a different response than excluding all large herbivores then it can be inferred that other wildlife are a source of the differences between exclusion and control treatments.

Crests and midslopes in the treatments are characterized by shallow, sandy, coarse soil, while footslopes are characterized by deep, sodic, duplex soil (Khomu and Rogers 2005; Grant and Scholes 2006). We sampled five deciduous and one evergreen species during the 2006/2007 wet season. The selected species were among the most abundant in the study area and included *Acacia grandicornuta* and *Euclea divinorum* on the footslopes and *Dichrostachys cinerea*, *Acacia exuvialis*, *Combretum apiculatum* and

Grewia flavescens on the middle slopes and crests (see Online Resource 1 for details of study area and species). Methods for measuring shoot or leaf growth were species-specific because of differences in shoot morphology. Both *D. cinerea* and the two *Acacia* species produce most of their new leaves in discrete clusters of leaves at nodes on old shoots or branches and therefore increase leaf area without growing internodes. Conversely, *C. apiculatum*, *G. flavescens* and *E. divinorum* produce all their new leaves on new shoots and therefore cannot increase leaf area without extending new internodes (Scogings et al. 2013a). The latter species experience more browsing by shoot biting than leaf nibbling and therefore experience more loss of biomass due to browsing compared to other species (Bond et al. 2001; Scogings et al. 2011).

Up to 10 new shoots and 10 new leaves on new shoots were selected on each of five trees per species in each exclusion treatment during the third week of November (two weeks after the first rainfall). Catenal position could not be regarded as a treatment because no species occurred across the catena. A 30 x 30 m grid was used to randomly locate plants. One tree of each study species was sampled in each of 10 randomly selected grid cells (five for the species inhabiting crests / midslopes and five for the species on footslopes) per treatment on condition that it was (1) closest to the grid-cell centre, (2) taller than 2 m (1.5 m for *G. flavescens* because it seldom grows taller than 2 m), and (3) neither obviously stressed by disease, disturbance or neighbours nor obviously growing in a nutrient enriched patch. If any of the conditions failed to be met for the closest tree, then the next closest tree that met the conditions was sampled. No tree was sampled if the closest suitable tree was further than 15 m from the grid-cell centre to avoid infringement of neighbouring cells. A similar study of trees shorter than 2 m was conducted in a different year (Scogings et al. 2013b).

The new shoots were selected by locating 10 old shoots (from a previous wet season) per tree. Each old shoot was 2-8 mm in basal diameter and 1.5-2.5 m above ground (>1 m for *G. flavescens*). Old shoots were marked by loosely twisting short pieces of thin, plastic-coated wire around the shoot base. Unique colour combinations were used to differentiate individual shoots. The most distal new shoot on each old shoot was then marked by painting a small spot of white “correction fluid” (Tippex[®]) at the base. In addition to marking new shoots on *A. exuvialis*, *A. grandicornuta* and *D. cinerea*, which had very few new shoots, one leaf cluster was marked on each marked old shoot. The leaf cluster with the longest leaf was marked with a small spot of Tippex[®] painted on the old shoot. The length of each marked new shoot was measured (nearest 0.1 cm) from the base of the shoot to the base of the apical bud. The length of the longest leaf on each marked new shoot and in each marked leaf cluster was measured (nearest 0.1 mm) from the base of the petiole to the apex of the midrib (simple leaves) or rachis (compound leaves). Each marked shoot that still had an intact apical bud in the first week of December, last week of January and last week of March was re-measured. The longest leaf on each marked shoot and in each marked leaf cluster was also re-measured.

Around each time that shoots and leaves were measured, six other plants of each species were located in each treatment by randomly selecting grid cells as described above. Leaf samples were collected from each plant by clipping branches 3-8 mm in diameter from the northern, sunlit half of the canopy between 1.5 and 2.2 m above ground, which was within the range of most browsers in the study area. For *G. flavescens*, the lowest permissible height for collection was reduced to 1.0 m because of its low stature. Branches were collected 1-4 hours after sunrise. Leaves that did not show signs of severe insect herbivory were randomly removed until a fresh mass of 30-

50 g was obtained for drying in an oven for 24 hours at 60 °C. Dried leaf samples were milled to pass a sieve with 1.0 mm apertures and were analysed for concentrations of condensed tannins ([CT]) (Porter et al. 1986), total polyphenols ([TP]) (Price and Butler 1977), Kjeldahl-N ([N]) (AOAC 1990) and phosphorus ([P]) (Murphy and Riley 1962). All concentrations were expressed in mg g⁻¹ dry mass. [CT] and [TP] were expressed as *Sorghum* tannin equivalents (STE) and Gallic acid equivalents (GAE), respectively. We note that variations detected by the CBSM assays may have several explanations, including changes in composition and reactivity.

Data analysis

Data were analysed separately for each species because we expected responses to be species-specific (Scogings et al. 2011). Trees were replicates. Therefore, pseudo-replication constrained the analysis (Underwood 1997). The shoot and leaf length data were averaged per tree, after which mean shoot or leaf length and associated standard error ($n = 5$) were plotted against time to identify distinct phases of growth (fast or slow). Chemical data were transformed to $\log_{10}(x)$, or $\log_{10}(\log_{10}(x))$, when this normalised the data. The ANOVA model containing the effects of herbivores (three levels), growth phase (two levels), and their interaction, was tested for [N], [P], [TP] and [CT] of each species, assuming a completely randomised design and type III sums of squares for unequal sample sizes. Tukey's HSD test was used to separate the means. Significance was declared when $P < 0.05$, while noteworthy trends were not ignored when $0.10 > P > 0.05$ (Underwood 1997).

Results

Two distinct phases of shoot or leaf growth were obvious, being rapid growth during the first 4-5 weeks of the wet season, followed by slow or negligible growth for the rest of the wet season in the deciduous species while the reverse pattern was observed in the evergreen species (Online Resource 2). Notwithstanding effects of growth phase and herbivores, species varied substantially in terms of measured chemical variables (Fig. 1). The only significant interaction between growth phase and exclusion treatment, suggesting that herbivore influence on plant chemistry differed between seasons, was for [CT] in *E. divinorum* (Fig. 2, Online Resource 3). While [CT] in *E. divinorum* was generally higher in the slow than the fast growth phase, it was lowest in the fast growth phase when large herbivores were present (Fig. 2). Marginally significant interactions were noted for both [CT] and [TP] in *A. grandicornuta* (Online Resource 3), suggesting that elephants tended to reduce [CT] and [TP] during the slow growth phase (Fig. 2).

Across all species, growth phase consistently affected both [N] and [P], which were lowest during the phase of slow growth, except in *E. divinorum* where the opposite was observed (Fig. 1, Online Resource 3). In *A. exuvialis* and *D. cinerea* [TP] was not significantly affected by growth phase (Fig. 1, Online Resource 3). In contrast, [TP] in *G. flavescens* and *E. divinorum* was higher during the slow than the fast growth phase, but the opposite was observed in *A. grandicornuta* and *C. apiculatum* (Fig. 1, Online Resource 3). In *A. exuvialis*, *D. cinerea*, *C. apiculatum* and *G. flavescens* [CT] was higher during the slow than the fast growth phase, but the opposite was observed in *A. grandicornuta* (Fig. 1, Online Resource 3).

Herbivores significantly affected [P] in *A. exuvialis* (Table 1), which was lower (mean = 2.35 mg g⁻¹; SEM = 0.090; *n* = 23) where all large herbivores were excluded,

compared to where either all large herbivores were present (mean = 2.76 mg g⁻¹; SEM = 0.151; *n* = 24) or all large herbivores except elephants were present (mean = 2.62 mg g⁻¹; SEM = 0.129; *n* = 22). In both *A. exuvialis* and *D. cinerea* [TP] was not significantly affected by herbivores (Online Resource 3). However, in both *C. apiculatum* and *G. flavescens*, [TP] was highest when all large herbivores were excluded, but in *E. divinorum* [TP] was higher when elephants were excluded than when no herbivores were excluded (Figs 2-3, Online Resource 3). In both *A. exuvialis* and *C. apiculatum* [CT] was highest when all large herbivores were excluded (Fig 3, Online Resource 3).

Discussion

One of our assumptions was that nutrient concentrations are highest, or CBSM concentrations are lowest, during phases of fast shoot or leaf growth. The higher nutrient concentrations and lower [CT] during the fast growth phase compared to the slow growth phase, except for *E. divinorum* and *A. grandicornuta*, supported the growth-differentiation balance hypothesis (GDBH) of plant defence (Hermes and Mattson 1992). However, [TP] in only two species (*G. flavescens* and *E. divinorum*) supported the GDBH by being lowest during the fast growth phase. Thus, some paradoxes were clear: (i) high [N] and [P] in the slow growth phase in *E. divinorum*, (ii) high or unchanged [TP] in the fast growth phase in four species, and (iii) high [CT] in the fast growth phase in *A. grandicornuta*. An explanation for reduced nutrient concentrations during the fast growth phase of *E. divinorum* late in the wet season is that evergreen species are known to withdraw nutrients from leaves to supply new apical buds (Pallardy 2008; Pornon et al. 2011). The ability of *E. divinorum* to grow late

in the wet season appears to be related to its occurrence on footslopes. A substantial proportion of rainfall infiltrates the coarse sandy soil on crests and eventually accumulates in the deep clay-rich subsoil of footslopes late in the wet season (Jacobs et al. 2007). The difference in the timing of water availability across the catena also supports observations that both *E. divinorum* and *A. grandicornuta* maintain positive radial stem growth in the dry season, while species on the sandy crests do not (Scogings 2011). Further evidence of increased water availability in footslope soils is the ability of *A. grandicornuta* to retain green leaves through most of the dry season in some years, despite being generally deciduous (Zululand/Sweden Kruger Browse Project, unpublished data). From our results we can infer that footslope species become potentially more palatable (increased N, reduced CT) as the wet season progresses, rather than becoming less palatable. Therefore, we postulate that footslope species are most likely to be eaten late in the season when high resource (water) availability on the footslopes would provide optimal conditions for plant recovery. Conversely, we would expect browsing to be concentrated on the crests early in the wet season when crest species are in optimal conditions to recover. However, support for the above hypothesis is absent and further research is needed.

High or unchanged [TP] or high [CT] during phases of fast shoot or leaf growth suggest that some deciduous woody species in savannas are less prone to C limitation than others, which is supportive of other studies (Palacio et al. 2008). Our observations imply either high C storage or high C assimilation capacities among deciduous species, which could be readily achieved in environments where light is not limiting (Scholes et al. 2003; Jolly and Running 2004). Positive associations between CBSM concentrations and growth occur when N is limited enough to affect growth rate but not photosynthesis, i.e., at moderate-high N limitation (Herms and Mattson 1992; Pizarro

and Bisigato 2010). We found high CBSM concentrations and fast growth at the start of the wet season, when N is most available (Scholes et al. 2003), suggesting that woody plants in the study area seldom experience high N availability. Therefore, high C reserves are important for woody plants in these ecosystems (Higgins et al. 2000; Scogings et al. 2011). Only *G. flavescens* consistently had high nutrient concentrations and low CBSM concentrations in the fast growth phase, suggesting it was not as N limited as the other species. Furthermore, CBSM synthesis requires N and any increase in N availability can lead to increases in both growth rate and C-based secondary metabolism (le Bot et al. 2009). For example, both *A. grandicornuta* and *D. cinerea* had high CBSM concentrations during the fast growth phase, which may be attributed to N₂-fixation.

Our second assumption was that when CBSM concentrations are low and resource availability is high, then CBSM concentrations are increased by browsing. The CBSM concentrations of the species characteristic of the crests and midslopes were reduced by large herbivores such that we inferred that elephants were not the main source of the effect. In contrast, the responses of the species characteristic of footslopes (discussed above) suggested the possibility that CBSMs were sometimes reduced by elephants. Nevertheless, our results corroborated the view that long-term browsing can maintain woody species in positive feedback loops characterised by N-rich or C-poor tissues (Fornara and du Toit 2007; Skarpe and Hester 2008). While long-term browsing generally reduced CBSMs, indicating C limitation among browsed woody plants in the study area, most of the woody species were generally able to accumulate CBSMs during phases of slow shoot or leaf growth during the wet season, indicating an absence of C limitation. We postulate that the reduction of CBSMs under browsing is a result of C being allocated to stimulated shoot or leaf growth (early in the wet season for most

species) (Fornara and du Toit 2007), but the increase in CBSMs during slow growth phases (late in the wet season for most species) is a result of C being allocated to functions other than the production of somatic or photosynthetic cells, such as the production of storage, transport, reproductive or defensive cells (Pallardy 2008). However, given that the negative effect of browsing on CBSMs persisted, C limitation ultimately prevailed among woody plants >2 m in height under long-term browsing in the study area.

The hypothesis that effects of large herbivores on nutrient or CBSM concentrations would be greater during phases of rapid shoot or leaf growth than during phases of slow growth was not supported. A significant interaction was only observed for [CT] in *E. divinorum*, which during the fast growth phase was most affected by elephant exclusion and therefore supported the hypothesis. *Euclea divinorum* was abundant on footslopes adjacent to a perennial river and experienced high rates of severe damage by elephants, such as branch/stem breakage (Scogings et al. 2012). Use of footslope vegetation increases when elephants increase the time they spend along perennial rivers during periods when other sources of drinking water are scarce (Grant et al. 2008). Rainfall during the 2006/2007 wet season was below the long-term mean (Scogings 2011), suggesting that elephant abundance and impact on the footslopes was intensified, which may have led to reduced defences resulting from C exhaustion (du Toit et al. 1990; Scogings et al. 2011). However, interactions between long-term and short-term effects require further research. Both the observed interaction in *E. divinorum* and the tendency for interactions in *A. grandicornuta* suggest that the effects of elephants on these footslope species are greatest late in the wet season. Conversely, we would postulate that the absence of significant interactions among the remaining

four species, which were abundant on the crests, was because browsing there was not as intense as on the footslopes (Engdahl 2008).

Conclusions

Our observations suggest that plant responses to resource availability (indicated by growth phase) and large herbivores in semi-arid African savannas are additive, rather than multiplicative. Elevated nutrients and reduced CBSMs generally occurred during phases of fast shoot or leaf growth among species characteristic of crests and midslopes, while species characteristic of footslopes responded conversely. In general, large herbivores reduced CBSMs, but did not affect nutrients. Overall, CBSMs in species on crests and midslopes were reduced by large herbivores other than elephants, while CBSMs in species on footslopes were reduced by elephants. Our conclusions should be considered with caution because spatial replication of treatments was not possible.

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Figure legends

Fig. 1 Mean concentrations of nitrogen (mg g^{-1}), phosphorus (mg g^{-1}), total phenolics ($\text{mg Gallic acid equivalents g}^{-1}$) and condensed tannins ($\text{mg Sorghum tannin equivalents g}^{-1}$) in mature leaves of *Acacia exuvialis* (AE), *Acacia grandicornuta* (AG), *Dichrostachys cinerea* (DC), *Combretum apiculatum* (CA), *Grewia flavescens* (GF) and *Euclea divinorum* (ED) plants ($n = 36$) at Nkuhlu, Kruger National Park, during fast and slow growth phases in the 2006/2007 wet season. Leaves were sampled between 1.5 and 2.2 m (1.0 – 1.5 m on *G. flavescens*). Asterisks indicate growth phases with significantly higher values ($P < 0.05$). Error bars are standard errors of the means

Fig. 2 Effects of large herbivores on mean concentrations of condensed tannins ($\text{mg Sorghum tannin equivalents g}^{-1}$) and total phenolics ($\text{mg Gallic acid equivalents g}^{-1}$) in mature leaves between 1.5 and 2.2 m on *Euclea divinorum* and *Acacia grandicornuta* at Nkuhlu, Kruger National Park ($n = 12$, but $n = 24$ for *E. divinorum* total phenolics). Letters indicate significant differences ($P < 0.05$) between treatments ($P < 0.10$ for *A. grandicornuta*). Error bars are standard errors of the means

Fig. 3 Effects of large herbivores on mean concentrations of total phenolics ($\text{mg Gallic acid equivalents g}^{-1}$) and condensed tannins ($\text{mg Sorghum tannin equivalents g}^{-1}$) in mature leaves between 1.5 and 2.2 m on *Acacia exuvialis* and *Combretum apiculatum*, and between 1.0 and 1.5 m on *Grewia flavescens*, at Nkuhlu, Kruger National Park ($n = 24$). Letters indicate significant differences ($P < 0.05$) between treatments. Error bars are standard errors of the means

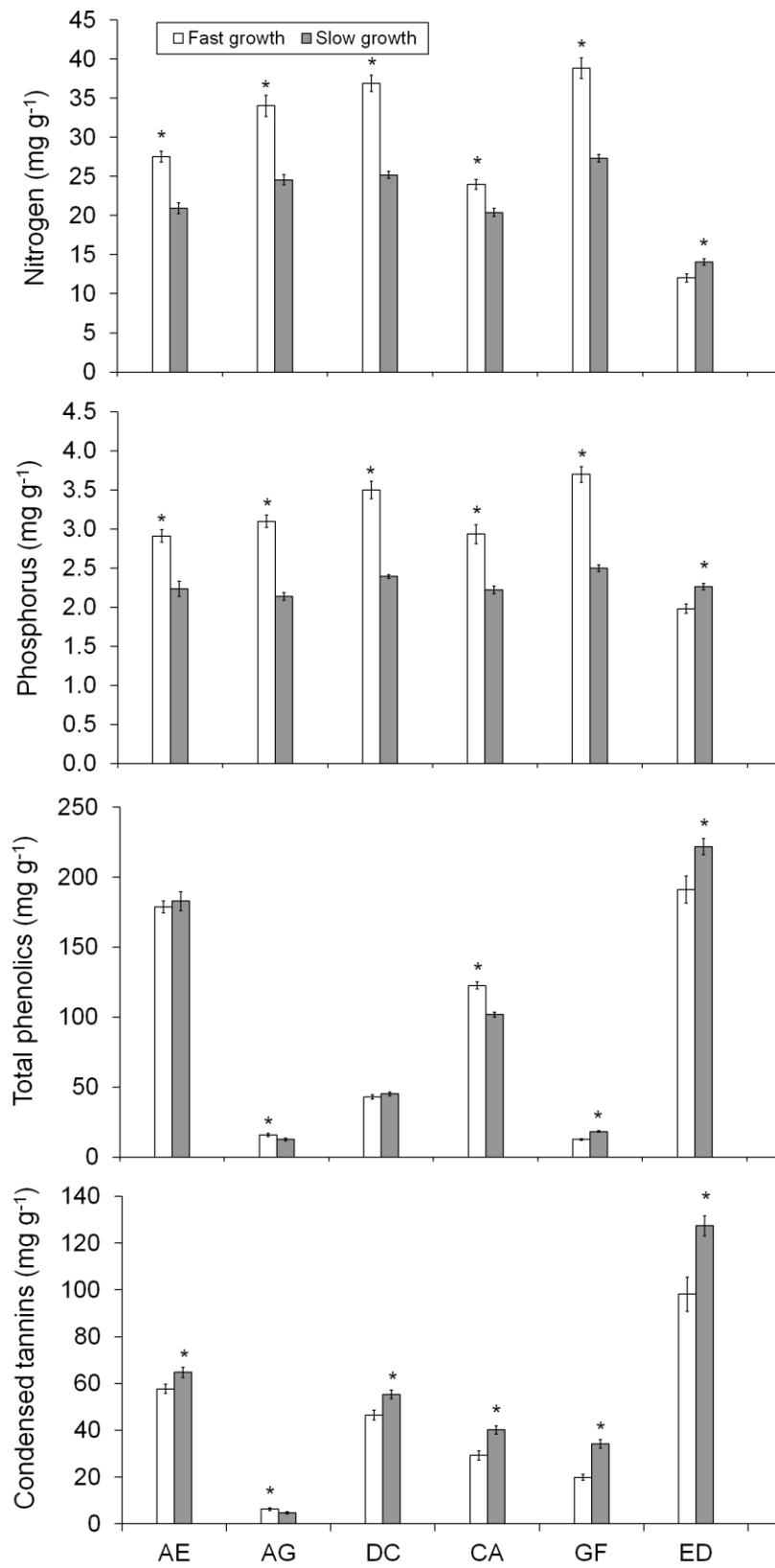


Fig. 1

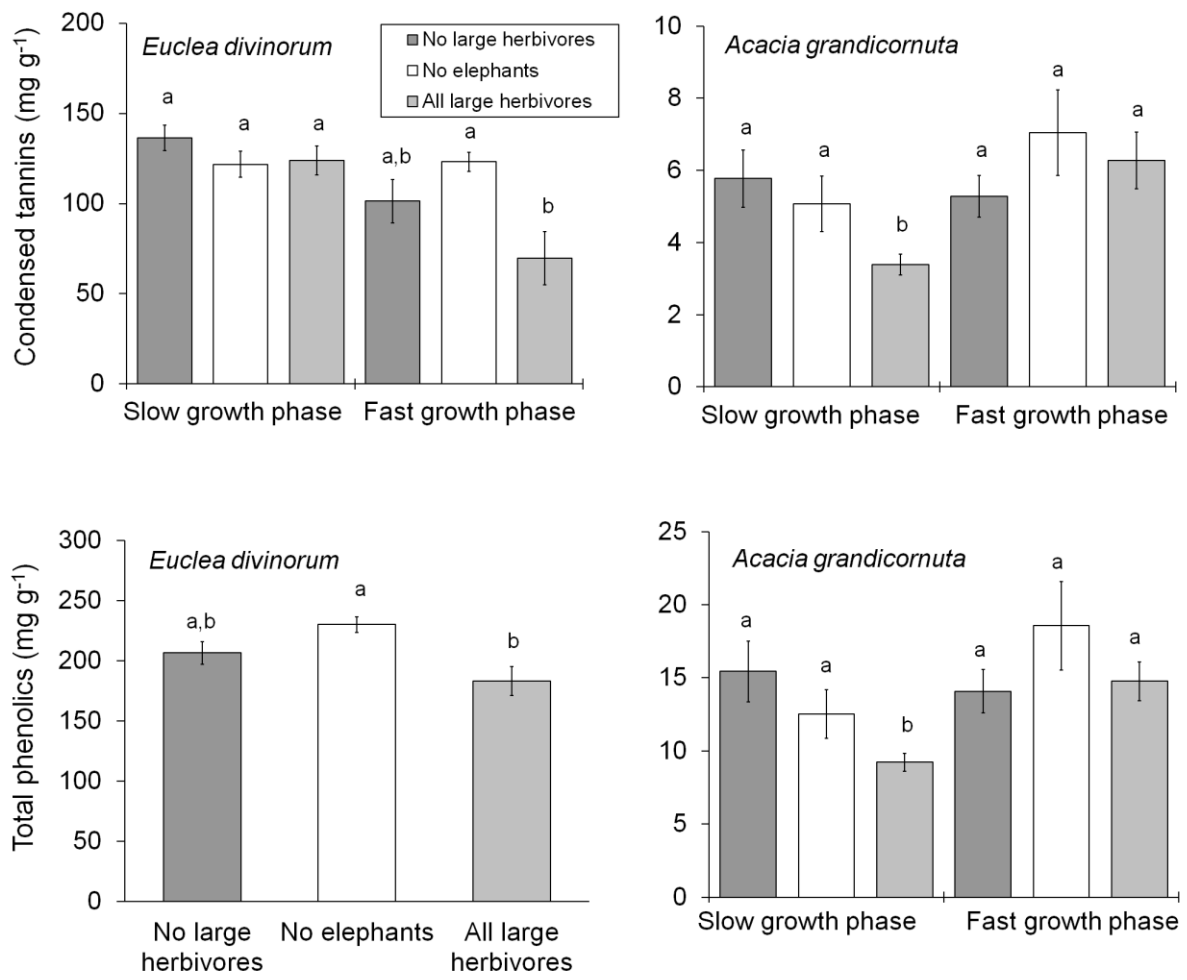


Fig. 2

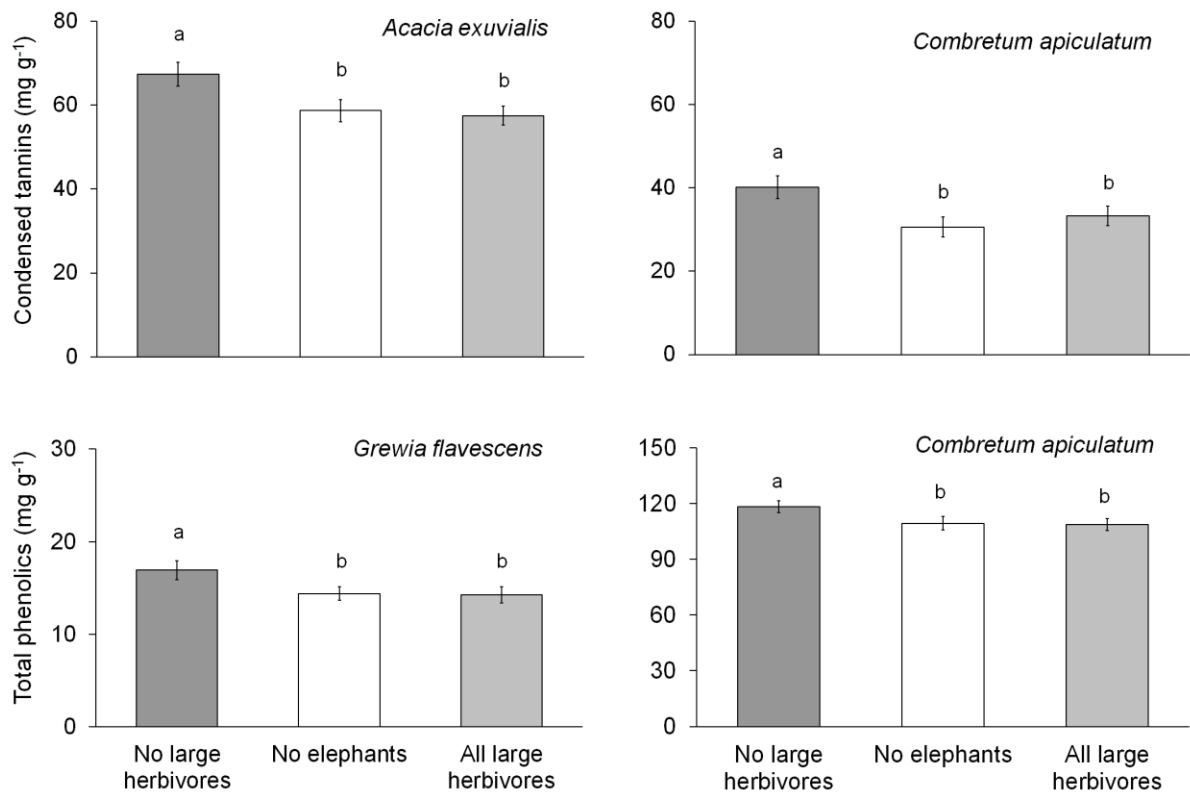


Fig. 3